Detection of Helicobacter pylori in head and neck cancer patients: Results from a prospective comparative study combining serology, PCR, and rapid urease test

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Abstract: BACKGROUND: The goal of this study was to evaluate prospectively the presence and impact of the gastric carcinogen Helicobacter pylori in the upper aerodigestive tract. Previous studies suggested it could represent a risk factor for head and neck squamous cell carcinoma. METHODS: Serology, rapid urease test, and quantitative polymerase chain reaction (qPCR) for H. pylori were performed in head and neck cancer patients (N=56) and cancer-free controls (N=90). Comparison between groups was done using logistic regression analysis. RESULTS: Rates of positive serology and rapid urease test did not differ between the two groups in logistic regression analysis (P=0.677 and P=0.633, respectively). Birth in a developing country and age above 50 years old were predictors of positive serology (P<0.001 and P=0.040, respectively). Using qPCR, no biopsy showed presence of H.pylori. CONCLUSION: This study challenges the concept that H. pylori may be a risk factor for head and neck squamous cell carcinoma.

DOI: https://doi.org/10.1002/hed.23958

Title

Detection of *Helicobacter pylori* in head and neck cancer patients: results from a prospective comparative study combining serology, PCR, and rapid urease test

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Acknowledgments

The authors would like to thank Anne Müller, PhD, Vital Wohlgensinger, PhD, and Réjane Morand, PhD, for their advice and technical support. We also would like to thank Amy Mavor, MA, for proofreading the manuscript and Nada Hafez, BSc, for her support in the statistical analysis.

Meeting presentation

This study was presented orally at the 68th Annual Meeting of Canadian Society of Otolaryngology – Head and Neck Society, May 11-13th 2014, Ottawa, ON, Canada.

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Running title

Helicobacter pylori in head and neck cancer

Keywords

Helicobacter pylori, head and neck cancer, serology, PCR, rapid urease test.

Abstract

BACKGROUND: The goal of this study was to evaluate prospectively the presence and impact of the gastric carcinogen *Helicobacter pylori* in the upper aerodigestive tract. Previous studies suggested it could represent a risk factor for head and neck squamous cell carcinoma.

METHODS: Serology, rapid urease test, and quantitative polymerase chain reaction (qPCR) for *H. pylori* were performed in head and neck cancer patients (N=56) and cancer-free controls (N=90). Comparison between groups was done using logistic regression analysis.

RESULTS: Rates of positive serology and rapid urease test did not differ between the two groups in logistic regression analysis (P=0.677 and P=0.633, respectively). Birth in a developing country and age above 50 years old were predictors of positive serology (P<0.001 and P=0.040, respectively). Using qPCR, no biopsy showed presence of *H.pylori*.

CONCLUSION: This study challenges the concept that *H. pylori* may be a risk factor for head and neck squamous cell carcinoma.

Introduction

Helicobacter pylori is a spiral-shaped gram-negative bacterium discovered by Marshall and Warren in 1984 [1]. It is recognized as an etiological agent of gastric cancer by the World Health Organisation (WHO) [2] and represents one of the most common infections worldwide [3]. The prevalence of *H. pylori* varies strongly between populations, being much higher in developing countries. Proposed risk factors include low socioeconomic status, increasing age, tobacco use, and infection of siblings [3].

The dominant infection route still remains controversial, while a human-to-human transmission seems probable [3]. *H. pylori* containing liquids are thought to infect new individuals orally, implying that *H. pylori* must be transiently present in the upper aerodigestive tract (UAT) [3]. The presence and potential impact of *H. pylor*i in the UAT is subject to debate [4, 5]. *H. pylori* has not only been associated with inflammatory conditions, which include aphthous ulcers [6, 7], chronic pharyngitis [8], and dental plaques [9, 10], but also with malignancy such as head and neck squamous cell carcinoma (HNSCC) [11-16].

We described in a previous retrospective pilot study using conventional polymerase chain reaction (PCR) that *H. pylori* can be found in the UAT of 38% of patients with proven gastric colonization [17]. Willing to validate the results of our pilot study, we conducted a prospective study combining serology, rapid urease test, and quantitative PCR (qPCR). A positive rapid urease test combined with a positive qPCR would indicate the presence of viable *H. pylori*, which would be confirmed by a positive serology. The primary goals of the study were to assess if *H. pylori* could be found in UAT samples of an unselected cohort of patients and if so, would be found more frequently among HNSCC patients. The latter would imply a potential carcinogenic role for *H. pylori* outside of the stomach.

Patients and Methods

After local ethics review board approval, we prospectively enrolled inpatients at the Department of Otolaryngology – Head and Neck Surgery of the University Hospital Zurich, Switzerland, from March 1st 2012 to September 30th 2012. Two groups of patients were included: the Tumor Group (TG) with patients undergoing panendoscopy for HNSCC, and the Control Group (CG) with patients undergoing tonsillectomy, thyroidectomy, parotidectomy, or

endoscopy for benign disease. All patients received an information form detailing the design and purpose of the study. The first author (GBM) was then responsible for discussing and settling any questions with the patients. After signing the informed consent form, the patients completed a survey to assess for their country of birth, tobacco use, alcohol consumption, and gastro-oesophageal reflux. Tobacco use was defined as a current daily consumption of cigars or cigarettes. Alcohol consumption was defined as a daily intake of more than 20 grams of ethanol at least five days a week. Gastro-oesophageal reflux was defined as troublesome heartburn occurring two days or more every week [(18)]. Exclusion criteria were age less than 18, history of *H. pylori* eradication, and previous radiation in the head and neck area.

A blood sample was drawn from each patient the day before surgery and sent for serologic examination using VIDAS *H. pylori* Ig6 EIA (BioMérieux, Marcy l'Etoile, France); the test was performed according to the manufacturer's instructions; the results were categorized as positive, negative, or indeterminate. During surgery, a mucosal specimen was obtained in the TG from the tumor (T) and from the surrounding healthy appearing tissue. The latter is further referred to as peritumoral (P). Peritumoral samples were taken approximately one centimeter away from the macroscopic tumor. In the CG, a sample was taken from the tonsillar surface in patients undergoing tonsillectomy. In the remainder of the CG, a biopsy was taken from the buccal mucosa. Following the operation, all samples were divided into two aliquots. The first part was used to perform a rapid urease test (Prontodry®, Medisafe©, Hamburg, Germany) according to the manufacturer's instructions. The second part was stored in Allprotect® Tissue Reagent (Qiagen©, Hombrechtikon, Switzerland) and frozen at -20°C until qPCR was performed (Figure 1).

Quantitative polymerase chain reaction (qPCR)

After genomic DNA isolation according to Secka *et al.* [(19)], qPCR was performed with Rotorgene® 6000 (Qiagen©, Hombrechtikon, Switzerland) with 25ng of each isolate. Dissociation and standard curves for all primers were generated. Quality control was carried out as recommended by MIQE Guidelines [(20)]. The reactions were carried out in triplicates for *H. pylori* on the *ureA* gene according to Schabereiter-Gurtner *et al.* (77-bp PCR product size; forward 5'-CGT GGC AAG CAT GAT CC AT-3'; reverse 5'-GGG TAT GCA CGG TTA CGA GT TT-3') [(21)]. GAPDH was used as endogenous control (216-bp PCR product size; forward 5'-AGG GCC CTG ACA

ACT CTT TT; reverse 5'-AGG GGA GAT TCA GTG TGG TG). GAPDH reactions were run in parallel to each *ureA* reaction to exclude PCR inhibition.

An external positive control was obtained using a stomach biopsy, for which *H. pylori* culture and histopathological staining were positive (courtesy of Ch. Gubler, MD, Clinic of Gastroenterology and Hepatology, University Hospital Zurich, Switzerland). Amplification occurring after threshold cycle (C_t) 35 and inconsistent among technical replicates was considered false positive[22]. The genomic DNA isolation and qPCR were performed by Microsynth©, Balgach, Switzerland, who were blinded on study group allocation and preliminary results.

Statistical analysis

For dichotomous variables, the two-sided chi-square test was used to compare proportions and phi-coefficient for correlations. For discrete variables showing normal distribution, means and standard deviations (SD) are given. Alternatively, median, first quartile (Q25), and third quartile (Q75) are indicated. For comparison of means, the t-test was used. Odds ratio (OR) and 95% confidence interval (95%CI) were calculated according to the Mantel-Haenzel method. A multivariate binary logistic regression model was used to compare the study groups after adjusting for significant variables. A parsimonious model was obtained by backwards elimination of irrelevant variables. For serology, analyses were always made twice, once assuming indeterminate cases to be positive, once to be negative. If not specified otherwise, the results of the alternate assumption did not differ significantly and are shown for the positive assumption.

An *a priori* power analysis was conducted with a power value of 80% and two-sided confidence level of 0.95. According to the available literature [23], we assumed a prevalence of approximately 30% in the TG and 10% in the CG, with a 2:3 ratio between TG and CG, and obtained a sample size of 50 and 75, respectively.

Statistical analyses were performed using SPSS® 21.0.0 software (IBM©, Armonk, NY, US) with the help of a biostatistician (see acknowledgements).

Results

In total, 146 subjects were included in the study. Baseline demographic data for the TG and CG are summarized in Table 1. A positive ELISA reaction from the blood sample was considered as positive serology. A mucosal

fragment eliciting a color change was considered a positive rapid urease test. For qPCR, amplification had to occur in all three technical replicates and before C_t=35 to be considered positive (for details see Patients and Methods). Rapid urease test and qPCR results for T and P biopsy did not differ notably (data not shown). Results from serology, rapid urease test, and qPCR were available for 133, 96, and 101 subjects, respectively. In univariate analysis, positive serology and rapid urease test did not differ significantly between the TG and the CG (Table 2). For qPCR, none of the samples were positive. Specifically, none of them showed amplification for *H. pylori* specific gene *ureA,* while the endogenous positive control for GAPDH and the external positive control were clearly positive at mean threshold cycle (C_t) of 22.3 (SD 1.79) and mean C_t of 28.5 (SD 0.04), respectively.

Using a logistic regression model controlling for potential confounding variables, no difference were seen for positive serology and positive rapid urease test between the two groups (*P*=0.677 and *P*=0.633, respectively). The variable "birth in a developing country" showed a highly significant relation with positive serology (*P*<0.001) in both univariate and multivariate analysis. Age above 50 years old also showed to be independent predictor of positive serology in multivariate analysis. The other variables – gender, smoking, alcohol consumption, and gastro-oesophageal reflux – were not significantly associated with positive serology (Table 3). A positive rapid urease test did not show any significant association with any of the variables (not shown).

The correlation between serology and rapid urease test was weak and did not reach statistical significance (phi=0.177 *P*=0.10).

Discussion

In a previous retrospective pilot study using conventional PCR, we found that 38% of patients with proven gastric colonization had presence of *H. pylori* in the upper aerodigestive tract (UAT). An association with malignancy could not be detected, nevertheless the study dealt with a small sample of patients [(17)]. Acknowledging the limitations of our pilot study and willing to validate its findings, we conducted this prospective comparative study using qPCR, rapid urease test, and serology.

Overall, we found *H. pylori* serology to be positive in approximately one-third of our patients, which is comparable to data from other western countries [(3)]. Head and neck cancer patients were not more likely to have positive serology than controls, in both univariate and logistic regression analysis. This result does not support an

association of *H. pylori* with HNSCC and is consistent with other serologic studies [24-26]. Two studies however reported *H. pylori* positive serology to be more frequent among head and neck cancer patients. Both of them compared cancer-free controls to laryngeal or hypopharyngeal cancer patients [11, 12]. The first study reported that *H. pylori* positive serology was associated with an odds ratio almost four times greater than smoking in head and neck cancer patients, suggesting unassessed confounding [11]. A potential confounder omitted in their multivariate regression analysis was increasing age, which is considered a risk factor for positive serology [3] and showed uneven distribution between the study groups (P=0.05) [11]. The second study did not perform multivariate analysis, and used the chi-square method although of limited sample size. Both factors render the interpretation of their results difficult [12, 27]. In our study, the only demographic variables significantly associated with a positive serology were birth in a developing country and increasing age, which is in accordance with epidemiologic data [3].

Rapid urease test was frequently positive in our study. However, the correlation between positive urease test and positive serology was weak. This is presumably due to the urease activity of bacteria other than *H. pylori* residing in the UAT, leading to non-specific positive rapid urease test [4]. Previous studies also reported low specificity of this test for extragastric samples [28]. Therefore, rapid urease test results from the UAT should be interpreted as "indicative of viable *H. pylori*" only if good concordance with serology and/or PCR can be observed.

Since *H. pylori* PCR can yield high false positive rates when performed on extragastric samples [21, 29], major attention was dedicated to the design of the qPCR experiment. We chose to use a validated qPCR protocol for extragastric samples, as described by Schabereiter-Gurtner *et al.* [21]. This protocol is highly specific as the target gene - *ureA* - was shown to have 100% homology with *H. pylori* genome only. It is also very sensitive since it can amplify bacterial DNA until 4 fg of DNA ($4x10^{-15}$g), which is equivalent to approximately 2.2 microorganisms [21]. Using this method, we failed to show *ureA* amplification in any of our test samples. This strongly suggests that *H. pylori* was not present in the UAT biopsy samples at the time of the biopsy. In the literature, reported PCR detection rates for *H. pylori* in the UAT range from 0% [5, 30] to 100% [16] in adults, with most studies situated between these two extremes [13-17, 31]. Many factors may be responsible for this discrepancy: firstly, the targeted genes and primers used to detect *H. pylori* vary widely. It was shown that some commonly used primers may not be fully specific, as they show complementarity with genomes of bacterial species other than *H. pylori* [21].

Secondly, even though specific PCR amplification proves that *H. pylori* DNA is present, it does not allow any conclusion on the viability of *H. pylori*. In fact, positive *H. pylori* culture has very rarely been reported in studies testing UAT samples, even if they showed high rates of positive PCR [14, 31]. Thirdly, it should be noted that studies with high PCR detection rates were mostly conducted in developing countries, where environmental exposure to *H. pylori* is higher [32]. Whether higher detection rates could also be explained by catching in-transit bacteria is yet to be elucidated.

H. pylori bacteria may also be detected in the UAT due to gastric contamination [33]. *H. pylori*-contaminated gastric fluids may reach the UAT in a retrograde fashion and lead to positive PCR results in the UAT. Gastro-oesophageal reflux and supine position would presumably encourage this phenomenon [33]. This factor could explain the apparent discrepancy with our pilot study, in which we amplified *H. pylori* DNA by PCR in 38% of the subjects testing UAT samples [17]. Unlike the present study conducted in a collective of head and neck patients with unknown *H. pylori* infection status, our pilot study only included patients with *H. pylori*-positive gastritis and coincidental UAT biopsy. Our database contained 2,830 *H. pylori*-positive gastric biopsies, yet only twenty-six patients met the inclusion criteria of the pilot study [17]. A larger collective of unselected patients in the present study may have resulted in few incidental individuals with active *H. pylori* gastric infection – and UAT contamination – leading to few positive qPCRs.

On a final note, we compared our results to the literature concerning oesophageal carcinoma, for which *H. pylori* infection was shown to be a protective factor [34]. The inverse relation between oesophageal cancer and *H. pylori* is thought to result from lower acidic gastric secretion – a consequence of *H. pylori* infection – and hence diminished chronic irritation of the oesophageal mucosa [35]. Since HNSCC shares many common etiological features with oesophageal squamous cell carcinoma, a positive relation between *H. pylori* infection and head and neck cancer appears unlikely [36]. Interestingly, the literature assessing the role of *H. pylori* in oesophageal cancer does not imply the presence of *H. pylori* in the oesophagus but investigates the *remote* effect of gastric *H. pylori* infection on the oesophageal mucosa [34]. Even in the assumption that *H. pylori* may be present in the UAT, it is questionable whether *H. pylori* can survive long enough outside the stomach to significantly impact carcinogenesis. Chronic

inflammation in the stomach, a significant pathway leading to gastric cancer [37], was shown to correlate consistently with bacterial loads of *H. pylori* [38].

In summary, our study provides evidence that *H. pylori* is not found frequently in the upper aerodigestive tract of a non-selected western population of head and neck cancer patients. Furthermore, we did not find head and neck cancer patients to show more likely *H. pylori* positive serology than cancer-free controls. Finally, rapid urease test showed non-specific results and should not be used alone for assessing extragastric samples. Although we do not believe that *H. pylori* is a major risk factor for HNSCC, further studies considering patients without history of nicotine consumption, alcohol use and negative human papilloma virus status could be of interest, particularly in populations from developing countries.

Conflict of interest statement

None of the authors report a conflict of interest.

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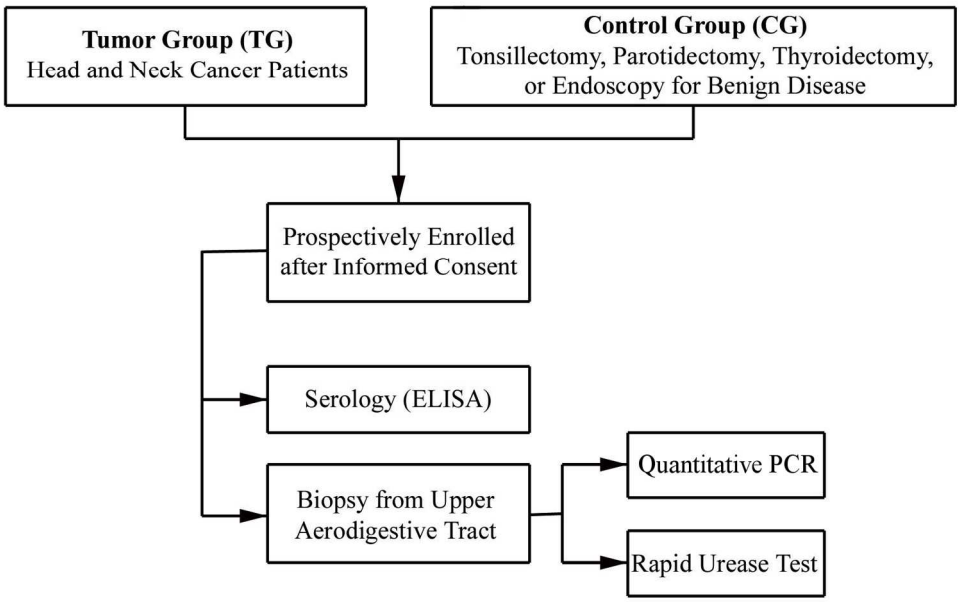

Figure 1: Overview of enrollment process and study design
637x423mm (72 x 72 DPI)

Tables

Table 1: Summary of patients' baseline characteristics

Variable	**Tumor Group** TG (N=56)	**Control Group** CG (N=90)	**All Patients** (N=146)	**TG vs. CG***
	Number of patients (% for each study group)			
Gender				
- Female	12 (21.4)	45 (50.0)	57 (39.0)	*P*<0.05
- Male	44 (78.6)	45 (50.0)	89 (61.0)	
Age				
- Median [Q25-Q75] †	63 [55-69]	37 [25-59]	55 [31-66]	*P*<0.05
Country Of Birth				
- High-income	51 (89.3)	70 (77.8)	120 (82.2)	*P*=0.12
- Developing	6 (10.7)	20 (22.2)	26 (17.8)	
Tobacco Use				
- Yes	33 (58.9)	43 (47.8)	76 (52.1)	*P*=0.17
- No	23 (41.1)	57 (52.2)	70 (47.9)	
Alcohol				
- Yes	44 (78.6)	19 (21.1)	63 (43.2)	*P*<0.05
- No	12 (21.4)	71 (78.9)	83 (56.8)	
Gastro-oesophageal Reflux				
- Yes	7 (12.5)	7 (7.8)	14 (9.6)	*P*=0.39
- No	49 (87.5)	83 (92.2)	132 (90.4)	
HNSCC Anatomical Site				
- Oral	8 (14.3)			
- Oropharynx	29 (51.8)			
- Larynx	14 (25.0)			
- Hypopharynx	5 (8.9)			
Type Of Intervention				
- Tonsillectomy		36 (40)		
- Parotidectomy		10 (11.1)		
- Thyroidectomy		30 (33.3)		
- Endoscopy for Benign Disease		14 (15.6)		

*: Chi-square test. *P* value for null hypothesis. †: first quartile (Q25) and third quartile (Q75).

Table 2: Main results by study group and comparison in univariate analysis

Method of detection	**Tumor Group** TG *Number of patients (% for each study group)*	**Control Group** CG	**All subjects**	**TG vs. CG***
Serology†				
- Positive	18 (34.6)	26 (32.1)	44 (33.1)	*P*=0.807
- Indeterminate	3 (5.8)	5 (8.2)	8 (5.4)	
- Negative	31 (59.6)	50 (61.7)	81 (60.9)	
Rapid urease test‡				
- Positive	16 (43.2)	16 (26.2)	32 (32.7)	*P*=0.264
- Negative	21 (56.8)	45 (73.8)	66 (67.3)	
Quantitative PCR§	0	0	0	

*: Chi-square test. *P* value for null hypothesis.
†: N= 133 [52+81]; ‡: N= 96 [35+61]; §: N=101 [38+63]; (N= total number of subjects for each method of detection. The numbers in brackets indicate the number of subjects for the TG and CG, respectively).

Table 3: Logistic regression model for positive serology

	*Unadjusted model**			*Adjusted model*†		
Variable	OR	95%CI	*P* value	OR	95%CI	*P* value
Gender						
- Female vs. Male	1.06	0.52-2.18	0.869			
Age						
- ≥50 years vs. <50 years	1.71	0.82-3.57	0.152	2.76	1.05-7.25	**0.040**
Country of birth						
- Developing vs. high-income	6.61	2.41-18.15	**<0.001**	8.00	2.67-23.9	**<0.001**
Tobacco use						
- Yes vs. No	1.59	0.78-3.22	0.197	1.39	0.64-3.01	0.402
Alcohol consumption						
- Yes vs. No	1.22	0.60-2.44	0.582			
Gastro-oesophageal Reflux						
- Yes vs. No	2.51	0.67-9.37	0.171	1.99	0.48-8.31	0.342
Study group						
- Tumor vs. Control	1.09	0.54-2.23	0.807	0.82	0.33-2.02	**0.677**

*: Unadjusted odds ratio according to Mantel-Haenzel method
†: Model adjusted for age, country of birth, tobacco use, and gastro-oesophageal reflux.
OR: odds ratio. 95%CI: 95% confidence interval. *P* value for null hypothesis.